

**Method for amplification of nucleic
acids of low complexity**

5 The present invention relates to a method for the amplification of nucleic acids.

Description

10 This invention relates to the fields of genetic engineering, molecular biology and computer science, and more specifically to the field of nucleic acid analysis based on specific nucleic acid amplification.

15 The matter of the present invention is a method for amplifying nucleic acids, such as DNA by means of an enzymatic amplification step, such as a polymerase chain reaction, specified for template nucleic acids of low complexity, e.g. pre-treated DNA, like but not limited to
20 DNA pre-treated with bisulfite. The invention is based on the use of specific oligo-nucleotide primer molecules to solely amplify specific pieces of DNA. It is disclosed how to optimize the primer design for a PCR if the template DNA is of unusually low complexity. Also, for the
25 optimal primer design it was considered that the treated template DNA is single stranded.

30 The amplification of nucleic acids relies mainly on a method called polymerase chain reaction (PCR). The PCR is based on the activity of the enzyme DNA polymerase, which is elongating primer molecules, which bind to the template DNA by adding dNTPs and hereby copying the template sequence (Saiki RK, Gelfand DH, Stoeffel S, Scharf SJ, Higuchi R, Horn T, Mullis KB and Erlich HA (1988)).
35 Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239 : 487-491). The

primer molecules are designed to specifically hybridize to those regions of the template DNA that define both ends of the amplificate. The forward primer binds to the 5' end of the sense strand of the amplificate, whereas the reverse primer binds to the 5' end of the reverse strand, hereby defining the starting points of the polymerase reaction and eventually determining the length of the amplificate.

Before the polymerase starts the template DNA gets denatured, this is usually done by a short cycle of heating the reaction mixture up to about 95°C, then cooling it down to the annealing temperature determined by the melting temperature of the primer molecules used and finally allowing the polymerase to elongate the annealed primers at its ideal working temperature for some minutes. This cycle is repeated several times each starting with the denaturation step. The primer molecules hybridize to the single stranded DNA. The forward primer is the starting molecule for a copy of the sense strand and the reverse primer is the starting molecule for a copy of the anti-sense strand.

These first copies will be of unspecific length, limited only by the polymerase's activity. However in the following cycle, the forward primer will also bind to the first copy of the anti-sense strand, the polymerase will take that copy as a template and will elongate the primer only as far as there is template DNA. Hereby the length of the second copy gets limited to the length defined by the first nucleotide of the second primer. In the following cycles more and more pieces of template DNA compete for the primer molecules and eventually the DNA amplificate of defined length will be the main product.

However, in the case of a bisulfite treated DNA the template DNA is single stranded. The bisulfite or similar treatment alters the original sequences on both strands such that these are not complementary to each other after the treatment. As a result no complementary strand to the target sequence exists. A first primer molecule binds to the one end of the single stranded target sequence. The polymerase elongates said primer and copies said target sequence. The second primer molecule cannot bind to the complementary, so called anti-sense strand, as it would in a standard PCR. Therefore the second primer molecule is designed to bind to the first copied sequence instead. More specifically it will bind to that part of the copied nucleic acid which is the complement to the other end of said target sequence.

The results of a PCR are highly depending on the choice of the ideal primer. The choice of a primer molecule must respect constraints permitting a correct amplification by PCR, fulfilling hybridization temperature conditions and auto- or hetero-hybridization prevention.

In other words, as any PCR requires two primer molecules to amplify a specific piece of DNA in one reaction the melting temperatures of both primers need to be very similar in order to allow proper binding of both at the same hybridization temperature. That is why most primer design programs require the user to define a preferred melting temperature or a permitted range of melting temperatures. This requirement becomes the limiting factor when designing primers for a so called multiplex PCR, as all primer pairs in use need to have the same or at least very similar melting temperatures. Additionally primers have to be very specific, in order to only amplify those pieces of DNA that are the target.

By providing the means for designing extremely accurate primer pairs for DNA hybridization procedures this invention relates to the so called PCR primer design. More specifically the body of this invention relates to the specific requirements of primers and therefore of primer design when using template DNA that consists of essentially only three different nucleotides and is single stranded. This is the case when using bisulfite treated DNA as a template, as it contains no cytosine other than the methylated cytosines in a CG dinucleotide and a rest of insufficiently treated and therefore untransformed non-methylated cytosines. The invention relates specifically to the primer design when using bisulfite treated DNA as template.

It would be obvious to an individual skilled in the art that the use of the primers as specified in this invention are not limited to nucleic acid amplification. Said primers can be used for several purposes, such as amplification, but also for nucleic acid sequencing or as blocking oligonucleotides during analysis of bisulfite treated DNA. Therefore the use of said primers is not limited to nucleic acid amplification but extends to all standard molecular biological methods.

Pairs of these primers are used to specifically amplify DNA from a small amount of sample DNA that consists of bisulfite treated DNA originating from a limited source of DNA like a bodily fluid or tissue sample.

DNA can occur methylated or non-methylated at certain positions and this information is relevant for the status of a genes transcription. The methyl group is attached to the cytosine bases in CpG positions. The identification of 5-methylcytosine in a DNA sequence as opposed to unmethylated cytosine is of greatest importance for example

when studying the role of DNA methylation in tumorigenesis. But, because the 5-Methylcytosine behaves just as a cytosine for what concerns its hybridization preference (a property relied upon for sequence analysis) its positions can not be identified by a normal sequencing reaction. Furthermore in a PCR amplification this relevant epigenetic information, methylated cytosine or unmethylated cytosine, will be lost completely.

This problem is usually solved by treating the genomic DNA with a chemical leading to a conversion of the cytosine bases, which consequently allows to differentiate the bases afterwards.

A tool most useful for analyzing DNA methylation is the bisulfite conversion of DNA that converts cytosine bases into bases showing a hybridization behavior as thymine bases. Hereby the DNAs complexity is reduced by a fourth.

Bisulfite conversion is the most frequently used method for analyzing DNA for 5-methylcytosine. It is based upon the specific reaction of bisulfite with cytosine which, upon subsequent alkaline hydrolysis, is converted to uracil, whereas 5-methylcytosine remains unmodified under these conditions (Shapiro et al. (1970) Nature 227: 1047). However, in its base pairing behavior, uracil corresponds to thymine, that is, it hybridizes to adenine; whereas 5-methylcytosine doesn't change its chemical properties under this treatment and therefore still has the base pairing behavior of a cytosine, that is hybridizing with guanine. Consequently, the original DNA is converted in such a manner that methyl-cytosine, which originally could not be distinguished from cytosine by its hybridization behavior, can now be detected as the only remaining cytosine using "normal" molecular biological techniques, for example, by amplification and hy-

bridization or sequencing. All of these techniques are based on base pairing which can now be fully exploited. Comparing the sequences of the DNA prior to and after bisulfite treatment allows an easy identification of those bases that have been methylated.

In the scope of this invention when it says "a nucleotide (...) was converted by the treatment..." this conversion is meant to be able to differentiate between methylated and un-methylated cytosine bases within said sample, as for example the conversion of un-methylated cytosine bases to bases which hybridize to adenine by the treatment with bisulfite.

An alternative method is to use restriction enzymes that are capable of differentiating between methylated and un-methylated DNA, but this is restricted in its uses due to the selectivity of the restriction enzyme towards a specific sequence.

An overview of the further known methods of detecting 5-methylcytosine may be gathered from the following review article: Rein T, DePamphilis ML, Zorbas H, Nucleic Acids Res. 1998, 26, 2255.

In terms of sensitivity, the prior art is defined by a method, which encloses the DNA to be analyzed in an agarose matrix, thus preventing the diffusion and renaturation of the DNA (bisulfite reacts with single-stranded DNA only), and which replaces all precipitation and purification steps with fast dialysis (Olek A, Oswald J, Walter J (1996) A modified and improved method for bisulfite based cytosine methylation analysis. Nucleic Acids Res. 24: 5064-6). Using this method, it is possible to analyze individual cells, which illustrates the potential of the method.

To date, barring few exceptions (e.g., Zeschnigk M, Lich C, Buiting K, Doerfler W, Horsthemke B (1997) A single-tube PCR test for the diagnosis of Angelman and Prader-Willi syndrome based on allelic methylation differences at the SNRPN locus. Eur J Hum Genet. 5: 94-8) the bisulfite technique is only used in research. Always, however, short, specific fragments of a known gene are amplified subsequent to a bisulfite treatment and either completely sequenced (Olek A, Walter J (1997) The pre-implantation ontogeny of the H19 methylation imprint. Nat Genet. 3: 275-6) or individual cytosine positions are detected by a primer extension reaction (Gonzalgo ML and Jones PA (1997) Rapid quantitation of methylation differences at specific sites using methylation-sensitive single nucleotide primer extension (Ms-SNuPE). Nucleic Acids Res. 25: 2529-31; WO 95/00669) or by enzymatic digestion (Xiong Z, Laird PW (1997) COBRA: a sensitive and quantitative DNA methylation assay. Nucleic Acids Res. 25: 2532-4).

Another technique to detect hypermethylation is the so called methylation specific PCR (MSP) (Herman JG, Graff JR, Myohanen S, Nelkin BD and Baylin SB (1996), Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. Proc Natl Acad Sci USA. 93: 9821-6). The technique is based on the use of primers that differentiate between a methylated and a non-methylated sequence if applied after bisulfite treatment of said DNA sequence. The primer either contains a guanine at the position corresponding to the cytosine in which case it will after bisulfite treatment only bind if the position was methylated. Or the primer contains an adenine at the corresponding cytosine position and therefore only binds to said DNA sequence after bisulfite treatment if the cytosine was unmethylated and has hence been altered by the bisulfite treatment so that it hybridizes to adenine.

With the use of these primers amplicons can be produced specifically depending on the methylation status of a certain cytosine and will as such indicate its methylation state. The present invention, however, does preferably not include CpGs in the primer sequence.

Another new technique is the detection of methylation via Taqman PCR, also known as MethyLight (WO 00/70090). With this technique it became feasible to determine the methylation state of single or of several positions directly during PCR, without having to analyze the PCR products in an additional step.

In addition, detection by hybridization has also been described (WO 99/28498).

Further publications dealing with the use of the bisulfite technique for methylation detection in individual genes are:

Grigg G, Clark S (1994) Sequencing 5-methylcytosine residues in genomic DNA. *Bioassays* 16: 431-6; Zeschnigk M, Schmitz B, Dittrich B, Buiting K, Horsthemke B, Doerfler W (1997) Imprinted segments in the human genome: different DNA methylation patterns in the Prader-Willi/Angelman syndrome region as determined by the genomic sequencing method. *Hum Mol Genet.* 6: 387-95; Feil R, Charlton J, Bird AP, Walter J, Reik W (1994) Methylation analysis on individual chromosomes: improved protocol for bisulphite genomic sequencing. *Nucleic Acids Res.* 22: 695-6; Martin V, Ribieras S, Song-Wang X, Rio MC, Dante R (1995) Genomic sequencing indicates a correlation between DNA hypomethylation in the 5' region of the pS2 gene and its expression in human breast cancer cell lines. *Gene* 157 : 261-4; WO 97/46705; WO 95/15373; WO 97/45560

For all those methods mentioned above, which are based on PCR amplification of bisulfite treated DNA, the biggest challenge is to design primers that are specific.

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THE PROBLEM AND ITS SOLUTION

There are a number of programs available on the market that offer to design primer pairs in order to amplify a piece of DNA in a PCR. Usually they require as input the
10 template DNA sequence, the preferred melting temperature T_M , the desired length of the amplificate and optionally the preferred length of the primer molecules.

However if a primer is required to bind specifically to
15 bisulfite treated DNA, the design of the primer molecule is especially difficult and those tools known in the art are not competent to design primers that lead to specific products. The following problems occur when dealing with bisulfite treated DNA instead of standard DNA:

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First, the sequence complexity of the bisulfite treated genome is reduced dramatically. Complexity in this context is meant to be a measure for the similarity of a given sequence to a random or stochastic sequence; the
25 more complex a sequence is the more it is similar to a random sequence. A reduced complexity of the genome means there are less degrees of variation. Where there are essentially only three different nucleotides rather than four, the probability of a sequence to occur twice in a
30 given length of sequence is much higher. For example, a primer molecule of 20 nucleotides in length is likely to be unique in the human genome, if it is not part of a repeat sequence: The human genome is known to consist of about 3×10^9 bases. There are $4^{20} \approx 10^{12}$ different ways
35 to form sequences of a length of 20 nucleotides, assuming equidistribution of the bases, which makes multiple oc-

currences of a given 20-mer (oligonucleotide of 20 nucleotides) extremely unlikely. However since there are only $320 \approx 3 \times 10^9$ different 20-mers possible over a 3-letter alphabet, this multiple occurrence cannot be excluded. In addition a bisulfite treated sequence, enriched in thymine in the sense strand and enriched in adenine in the reverse complementary strand, will contain more repeats and regions of general low complexity.

Another way to enhance or guarantee uniqueness of primer and/or oligo molecules is to estimate their expected frequency in the genome based upon a Markov model of order n for the human genome or to check their uniqueness explicitly by counting their exact occurrence. The estimation based upon the Markov model relies upon the determination of the probabilities of all 4^n n -mers (oligo molecules of n nucleotides) in the human genome or in all amplicates which are used in the hybridization and the conditional probabilities of all four bases given these n -mers. The primer pairs will be constructed from forward and reverse oligos which lie within an appropriate distance to each other and which have minimal individual expected occurrence elsewhere in the genome.

A second challenge in primer design for bisulfite treated DNA is that the melting temperature T_M of a bisulfite DNA primer of a certain length is typically lower than the melting temperature T_M of a standard primer containing cytosines. This is due to the fact that every cytosine in a bisulfite treated DNA is - after amplification by PCR - replaced by thymine. Cytosine binds its corresponding base guanine via three hydrogen bonds, whereas thymine binds its corresponding base adenine via two hydrogen bonds only, leading to a generally weaker binding, a lower T_M .

A third problem arises from the fact that bisulfite treated sequences are not only lacking cytosines but are also thymine-rich. Thymine also hybridizes unspecifically with guanine. This makes mismatching (unspecific binding of a primer to a sequence not identical) of a primer designed for bisulfite treated DNA much more likely than mismatching of a standard primer consisting of four different nucleotides.

It is the aim of this invention to overcome these problems, which are specific for primer based amplification of bisulfite treated DNA.

For a so called "multiplex PCR" it becomes especially difficult to design primer pairs. This expression is used to describe an experiment in which several different pieces of DNA are amplified simultaneously, in one reaction vessel and at the same time. Obviously this saves a lot of effort and time and is as such a basic requirement for high throughput assays based on PCR amplification. An overview on the state of the art concerning multiplex PCR is given by Henegariu et al. (Henegariu O, Heerema NA, Dlouhy SR, Vance GH and Vogt PH (1997) Multiplex PCR: Critical Parameters and Step-by-Step Protocol. BioTechniques 23: 504-511), who offer a step-by-step protocol on how to tackle multiplex PCR problems. However, the possibility of a special primer design is not mentioned in this article.

To ensure that the multiplex PCR works and the multiple products are amplified indeed usually a gel electrophoresis of the reaction mixture is performed. The products get separated due to their different sizes. Unfortunately, the ability of agarose gel electrophoresis to distinguish the products is slightly limited. However, it is possible to test for different product sizes with the

means of a fragment analyzer, which is much more accurate and able to distinguish product sizes of one base difference. Hence different product sizes are no longer a requirement to be considered in the primer design for a multiplex PCR.

In patent WO 01/94634 a method for a multiplex PCR using at least two primer pairs is described that consists of basically a two step amplification procedure wherein one step is referred to as pre-amplification. After pre-amplification (by means of PCR) with a number of primer pairs the sample gets divided into as many portions as there are primer pairs. At least one (and preferably only one) of the previously used primer pairs is added. This method doesn't relate in any way to the selection or design of primer molecules described herein.

In an article by Shuber et al. (Shuber AP, Grondin VJ and Klinger KW (1995) A simplified procedure for developing multiplex PCRs. Genome Res 5 (5) : 488-493) regarding multiplex PCR, the authors suggest to use primers, which contain a 3' region complementary to sequence specific recognition sites and a 5' region of a defined length of 20 nucleotides each. The authors claim that they could establish identical reaction conditions, cycling times and annealing temperatures for any PCR primer pair following those requirements.

In several recent papers successful multiplex PCRs have been established. For example, Becker et al. have reported the development of a multiplex PCR reaction for the detection of multiple staphylococcal enterotoxin genes, which uses individual primer sets for each toxin gene (Becker K, Roth R and Peters G (1998) Rapid and specific detection of toxigenic Staphylococcus aureus : use of two multiplex PCR enzyme immunoassays for amplifica-

tion and hybridization of staphylococcal enterotoxin genes, exfoliative toxin genes, and toxic shock syndrome toxin 1 gene. J. Clin. Microbiol. 36: 2548-2553). This has been developed even further by Monday and Bohach, by increasing the number of primer pairs applied in one reaction up to about 10 in order to have one assay to amplify all of the characterized enterotoxin genes. This still required a unique established primer pair for the detection of every individual gene (Monday SR and Bohach GA (1999) Use of multiplex PCR to detect classical and newly described pyrogenic toxin genes in staphylococcal isolates. J. Clin. Microbiol. 37: 3411-3414).

In another paper by Sharma et al. a method for a one-vessel-multiplex PCR is described wherein each of six chosen primer pair consists of one identical universal forward primer, based on a highly conserved region of those genes of interest and one reverse primer, specific for each individual gene. As such the assay leads to a rapid amplification of a family of genes, which all have a conserved region in common. It is designed to detect presence or absence of certain genes in an unknown mixture. No further information is given about the primer design, apart from saying that they were designed by alignment of published DNA sequences. This is certainly not the only requirement though, as one big limitation of the method is the need of getting PCR products of different sizes in order to identify those in the end (Sharma NK, Rees CED and Dodd CER (2000) Development of a single-reaction multiplex PCR toxin typing assay for Staphylococcus aureus strains. Applied and Environmental Microbiology 66 (4) : 1347-1353).

In the patent application WO 01/36669 a method is described which uses a similar approach for the controllable amplification of a higher number of sequences in selec-

ting one randomly chosen reverse primer that hybridizes unspecifically and a number of specific forward primers to amplify a group of sequences. As the reverse primer is labeled all products formed will be labeled as well. By hybridizing said amplicons towards immobilized detection oligos, which are able to differentiate the products, it will be easy to see which products have been amplified and herein the presence or absence of said sequences in the mixture can be determined.

The big disadvantage in all these methods is that every primer pair needs to be established individually first to ensure that a PCR product of the expected size was produced and that no additional or nonspecific products are generated. Once the specificity of the primer pairs had been determined, PCR conditions, buffers, and primer concentrations need to be optimized to establish conditions under which the primer molecules can be combined into one single PCR reaction without affecting the ability of the primer pairs to generate a gene specific amplicon.

A more recently published approach by Nicodeme and Steyaert describes the conditions required for multiplex PCR and suggests an algorithm to automatically select for primer pairs (Nicodeme P and Steyaert JM (1997) Selecting optimal oligonucleotide primers for multiplex PCR. Proc. Int. Conf. Intell Syst Mol Biol; 5 : 210-213). In this approach the conditions for pre-selecting primer pairs for a successful one locus amplification (singleplex PCR conditions) are rather broad. The three basic requirements are the pairing distance between a forward and a reverse primer, the condition of non-palindromicity of a primer, and the condition that the 3' end of a primer must not be reverse complementary to any of the other primers sequence. This selection is done with the help of a typical primer design program called PRIMER. However,

PRIMER is a two step program, and in this approach the new method to design primers for a multiplex PCR takes the output from step 1 as input, which is a list of possible forward and a list of possible reverse primers for every amplificate.

The only further selection criteria for the multiplex PCR primers are the absence of the reverse complementarity of their 3' end towards the other primer sequences in the experiment. A second critical factor considered here is the GC versus AT ratio. To some extent it is this ratio that determines the melting temperature of a primer pair. The authors suggest to limit the GC/AT ratio to be inside a given range which would enable the simultaneous hybridization of several primer pairs at one reaction temperature. The final requirement is the electrophoresis distance, determined by the tool that is used to differentiate the PCR products in, for example, a gel electrophoresis. This most common method requires the products to be of different sizes. The whole concept of this method also requires to have a pool of possible primer pairs for each amplicon.

The design of suitable primers for a multiplex PCR on bisulfite treated DNA is an even greater challenge. The low complexity of the DNA, being reduced to essentially three different bases rather than four different bases, requires an extra careful selection of primers to avoid mismatching and unwanted amplification.

In the scope of this invention the word "mismatching" corresponds to the situation when the alignment of two sequences which are essentially complementary reveals positions in one of the sequences where the nucleotide base does not align with its corresponding base but a different one. The corresponding or complementary base pairs are adenine and thymine, cytosine and guanine,

are adenine and thymine, cytosine and guanine, uracil and adenine. For example, a cytosine that aligns with a thymine in its otherwise complementary sequence creates a mismatch of one base or nucleotide.

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Accordingly "base mismatches" refers to the situation of a base mismatching with another as explained above, respectively "one or more base mismatches" refers to one or more bases (in a given sequence) that cannot be aligned with their corresponding bases.

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Also, when the alignment reveals single nucleotide gaps in one of the aligned sequences this is understood under the term "mismatch" in the scope of this invention.

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A 'gap' is to be understood as follows: If an alignment reveals that, in order to get the highest number of corresponding base pairs aligned, some bases are lacking a corresponding base in its otherwise complementary sequence, this is called a gap. Such a gap can have a length of one or more nucleotides.

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To solve the problems mentioned above we invented a method consisting of several steps that is applicable for the amplification of nucleic acids in singleplex as well as in multiplex PCR experiments.

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SUMMARY OF THE INVENTION

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The method is comprised of the following steps:

Firstly, the nucleic acid sample containing the region of interest, which is to be amplified, is isolated. Secondly, this nucleic acid sample is treated in a manner that differentiates between methylated and un-methylated cytosine bases within said sample. Thirdly, a reaction mixture is set up containing a) the treated template nu-

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cleic acids, carrying the region of interest (also called: target nucleic acid) that is to be amplified, b) specified oligo-nucleotide primers, c) an enzyme capable of amplifying said nucleic acids in a defined manner, d) the necessary nucleotides required for the nucleic acid synthesis and e) a suitable buffer.

Said specified oligo-nucleotide primers are characterized in that

their sequences each reach a predefined measure of complexity (as described in detail below) every possible combination of two primer molecules in said reaction mixture has a melting temperature below a specified threshold temperature

none of the possible combinations of two primer molecules in said reaction mixture leads to the amplification of an additional unwanted product as determined by virtual testing for amplification.

In the last step of the method said amplified target nucleic acid is detected by means commonly used by one skilled in the art.

The invention is composed of a method for the amplification of nucleic acids comprising the following steps of isolating a nucleic acid sample, treating said sample in a manner that differentiates between methylated and unmethylated cytosine bases within said sample, amplifying at least one target sequence, within said treated nucleic acid, by means of enzymatic amplification and a set of primer molecules, wherein said primer molecules are characterized in that

a) each primer molecule sequence reaches a predefined measure of complexity, b) every combination of any two primer molecules in the set has a melting temperature below a specified threshold temperature and c) every combi-

nation of two primer molecules, under conditions allowing for one or more base mismatches per primer, does not lead to the amplification of an unwanted product when virtually tested using the treated and the untreated sample nucleic acids as template and the last step of detecting said amplified target nucleic acid.

More detailed description of the method:

The method is comprised of the following steps:
In the first step of the method, the nucleic acid sample, which contains the region of interest that is to be amplified, must be isolated from tissue or cellular sources. Such sources may include at least one cell, but usually several cells, cell lines, histological slides, bodily fluids, or tissue embedded in paraffin.

In a preferred embodiment of this invention the nucleic acid sample is isolated from a bodily fluid, a cell culture, a tissue sample or a combination thereof.

For example a certain kind of organ sample from a patient or an animal can be used to extract genomic DNA by the usually applied methods. Preferably, in this invention DNA is extracted from a tissue sample or a biological fluid like blood, serum, urine or other fluids. 'Bodily fluid' herein refers to a mixture of macromolecules obtained from an organism. This includes, but is not limited to, blood, blood plasma, blood serum, urine, sputum, ejaculate, semen, tears, sweat, saliva, lymph fluid, bronchial lavage, pleural effusion, peritoneal fluid, meningeal fluid, amniotic fluid, glandular fluid, fine needle aspirates, nipple aspirate fluid, spinal fluid, conjunctival fluid, vaginal fluid, duodenal juice, pancreatic juice, bile and cerebrospinal fluid. This also includes experimentally separated fractions of all of the

preceding. 'Bodily fluid' also includes solutions or mixtures containing homogenized solid material, such as feces.

5 The nucleic acids may include DNA or RNA. Isolation may be by means that are standard to one skilled in the art, this includes for example extraction of DNA with the use of detergent lysates, sonification and vortexing with glass beads. An example is the extraction of DNA from a
10 piece of a plant, like a leave or fruit. Once the nucleic acids, like genomic double stranded DNA, have been extracted they are used in the analysis.

15 In a preferred embodiment of this invention the nucleic acid sample is comprised of plasmid DNA, BACs (bacterial artificial chromosomes), YACs (yeast artificial chromosomes) or genomic DNA.

20 In another especially preferred embodiment of this invention the nucleic acid sample is comprised of human genomic DNA. It is preferred that the nucleic acids are of human origin.

25 In the second step, this nucleic acid sample is treated in a manner that differentiates between methylated and un-methylated cytosine bases within said sample. Cytosine bases which are unmethylated at the 5'-position are converted to uracil, thymine, or another base which is dissimilar to cytosine in terms of hybridization behavior.
30 This will be understood as 'treatment' hereinafter. The method most commonly used so far is the so called bisulfite treatment.

35 This step is of essential meaning to the process as it translates the methylation pattern of said nucleic acids into a pattern that is something like an imprint of the

methylation status itself. It contains essentially the same information but the pre-treated nucleic acids are no longer sensitive to amplification via PCR. Amplification via PCR does not differentiate between methylated and un-methylated cytosines and therefore leads to the loss of this level of information. The original methylation status however can be deducted whenever the described pre-treatment had been performed prior to the amplification step. Hence any means suitable to differentiate between a methylated and an un-methylated cytosine base are applicable, as long as the modified bases are still capable of being amplified by enzymatic means after treatment.

It is a preferred embodiment of this invention that said sample is treated by means of a solution of a bisulfite, hydrogen sulfite or disulfite. A treatment of genomic DNA as described above is carried out with bisulfite (hydrogen sulfite, disulfite) and subsequent alkaline hydrolysis which results in a conversion of non-methylated cytosine nucleobases to uracil or to another base which is dissimilar to cytosine in terms of base pairing behavior.

In the third step of this method, a reaction mixture is set up containing a) the treated template nucleic acids, comprising the region of interest (also called target nucleic acid) that is to be amplified, b) specified oligonucleotide primers, c) an enzyme capable of amplifying said nucleic acids in a defined manner, for example a polymerase, d) the necessary nucleotides required for the nucleic acid synthesis and e) a suitable buffer. The template nucleic acid contains at least one target nucleic acid, which is amplified in the reaction. One primer molecule of the at least one primer pair in the reaction mixture is capable of binding to the 3' end of one specified target nucleic acid. The first primer binds to the

3' end of the target sequence, this primer is elongated and a complementary sequence to the target sequence is made. The polymerase stops to elongate unspecifically. The next cycle starts by thermally denaturing the now
5 double stranded template nucleic acid into single stranded template nucleic acids. This is followed by the next phase of annealing when both primer molecules specifically bind to the target nucleic acid and its complementary strand. The second primer is identical to the 5'
10 end of the target molecule. It doesn't bind to the target sequence itself but to said complementary nucleic acid to the target sequence, as soon as this is denatured from the template.

15 The process is finished by the actual amplification phase at a slightly lower reaction temperature, during which the enzyme, for example the polymerase elongates the primer as a complementary sequence to the target nucleic acid. The polymerase elongates this second primer by using the first copy as template until the end of said copied nucleic acid is reached. That way an identical copy
20 to the original single stranded target nucleic acid is created. Hence, the length of the amplificate is determined by choosing the two primers.

25 The elongation products, being complementary to each other and hereby building a double stranded version of the target nucleic acid, serve as additional targets for the primer molecules binding in the next cycle of amplification.
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Essentially step 3 of the method is comprised of amplifying at least one target sequence, within said treated nucleic acid, by means of enzymatic amplification and a set
35 of primer molecules.

Said primer molecules used in said method are characterized in that they, in addition to fulfilling all the usual requirements towards a PCR primer as will be specified in more detail later, also fulfill the following requirements:

Firstly, the sequence of each primer molecule used in step 3 of this method reaches a predefined measure of complexity.

In a preferred embodiment of this method the primer molecules are reaching a certain value of linguistic complexity. A notion and a measure of linguistic complexity has been introduced by Trifonov in 1990 and has been used for analysis of nucleotide sequences before (Trifonov, EN (1990) Making sense of the human genome. In Structure & Methods. Vol 1 pp 69-77 (eds. Sarma, RH and Sarma MH, Adenine Press, Albany, US). The linguistic complexity technique allows a calculation to be made of the structural complexity of any linear sequence of characters irrespective of whether the text is cognized or presently undeciphered. The sequences are compared exclusively from the point of view of their structural complexity with no reference to the meaning of the texts. In 1997 Trifonov published how the linguistic complexity of nucleosomal sequences is defined (Bolshoy, A; Shapiro, K; Trifonov, E and Ioshikhes I. (1997) Enhancement of the nucleosomal pattern in sequences of lower complexity. NAR 25 (16): 3248-3254). Quote: "The linguistic complexity measure exploits the major distinguishing feature between natural nucleotide sequences and uniformly random ones: the repetitiveness of the natural sequences, i.e. the frequent repetition, not necessarily a tandem one, of some oligonucleotides ("words"), while others are avoided. (...) Complexity can be directly calculated as the extent to which the maximal possible vocabulary (all word sizes

considered) is utilized in a given strength of sequence (...)."

5 In another preferred embodiment of this method said measure of complexity is set by the so called Shannon entropy (Shannon, C E, (1948) A Mathematical Theory of Communication, University of Illinois Press, Urbana). This is the most common measure to assess the information content (in a technical, non-semantic meaning) of linear information
10 carriers. It attributes the maximal value (which can be chosen to be 1 without restrictions) to sequences where all symbols (characters) occur at equal probability and a value of 0 to sequences consisting of just one repeated symbol (character, letter). A derived and more general
15 measure is the higher order Shannon entropy which attributes maximal value to sequences where all its subsequences occur at equal probability and a value of 0 or close to 0 to sequences consisting of periodic repetitions of short subsequences. The practical determination
20 of the (higher order) Shannon entropy however is limited by the finite lengths of sequences which often does not permit a precise estimation of the probability distribution of their constitutive symbols.

25 Further possible measures are for example the Lempel-Ziv complexity (Lempel, LB and Ziv, J (1976) On the complexity of finite sequences. IEEE Trans. Inf. Theory IT-22, 75-81) , the grammar complexity (Ebeling, W; Jimenez-Montano, MA (1980) On Grammars, Complexity and Information Measures of Biologicial Macromolecules. Mathematical Bioscience 52, 53-71), the algorithmic complexity
30 (Chaitin, 1990) and the conditial entropy.

35 Secondly, said primer molecules are also characterized in that every possible combination of any two primer molecules, in the set, has a melting temperature below a

specified threshold temperature. That way the accumulation of dimers caused by the binding of two primer molecules to each other in said reaction mixture is excluded. The number of primer pairs used in that step can be any
5 between one and n, leading to one or n amplicates respectively (n being a natural number).

As mentioned in the text the word "dimer" refers to a secondary structure formed by the hybridization of two
10 primer molecules to each other.

As referred to in the text 'melting temperature' refers to the temperature at which 50% of the nucleic acid molecules are in duplex and 50% are denatured under standard
15 reaction solution conditions.

Some primer design tools disqualify a primer if, besides the target sequence, a second identical sequence can be found in the template. However, due to the higher probability of a bisulfite primer to mismatch with non-
20 identical bisulfite treated DNA, it is an embodiment of this invention that only those primers are allowed to be used in said amplification method, for which no sequence homology can be found, to the extent that even those sequences that are different and/or mismatching in several
25 nucleotides are excluded. However, this would exclude primer molecules unnecessarily. Therefore they are only excluded if two primer molecules match to the template in a distance allowing for the amplification of an unwanted
30 product. This test is performed by means as, for example, the Electronic PCR. Electronic PCR (e-PCR) is an in silico virtual PCR carried out in order to assess the suitability of primer molecules prior to in vitro PCR. In the scope of this invention this testing will be called
35 'virtual testing' and it will be referred to as "virtually tested" or "virtually testing".

Thirdly, the primers used in step 3 of this invention are characterized, in that every possible combination of two primer molecules, in said reaction mixture, does not lead to the amplification of an additional unwanted product, when virtually testing for amplification using the treated and the untreated nucleic acid sample as template, even under conditions allowing for at least one base but not more than 20% of the total number of bases per sequence mismatching per primer. In the scope of this invention it is to be understood that those primer molecules are considered to bind to the template for which a template sequence exists that is in at least 80% of its nucleotide sequence identical to the target sequence the primer originally has been designed for. For example, a primer molecule of 50 nucleotides length is considered to still hybridize to a template sequence that differs in less than 11 nucleotides (= is identical in at least 80% of its nucleotide sequence) from the according target sequence. If a match is considered to be possible it has to be tested whether this match would lead to the amplification of an unwanted product. This can be done with the use of a program similar to e-PCR (see below).

Especially preferred is an embodiment of said method wherein the ability of said primer molecules to amplify an unwanted product is tested by means of in silico PCR, taking as template nucleic acid the coding strand of the treated sample, the non-coding strand of the treated sample and both of the strands of the untreated sample. It is especially preferred to perform the virtual testing with a tool like electronic PCR on the pretreated, preferably bisulfite treated, template sequence consisting of the treated sense and the treated anti-sense strand, and on the unconverted template.

Furthermore it is preferred that this treatment is bisulfite treatment and hence the nucleic acid template is the bisulfite converted coding strand of the human genome, the bisulfite converted non-coding strand of the human genome and both of the strands of the untreated human genome. Preferred is an embodiment of said method wherein the ability of said primer molecules to amplify an unwanted product is tested by means of electronic PCR, hereby taking as template nucleic acid the bisulfite converted coding strand of the human genome, the bisulfite converted non-coding strand of the human genome and both of the strands of the untreated human genome.

It is preferred that the number of mismatches allowed for when virtually testing the amplification of unwanted products according to step 3 c) of the invention is less than 20% of the number of nucleotides of the primer.

It is also preferred that the number of mismatches allowed for when virtually testing the amplification of unwanted products according to step 3 c) of the invention is less than 10% of the number of nucleotides of the primer.

It is especially preferred that the number of mismatches allowed for when virtually testing the amplification of unwanted products according to step 3 c) of the invention is less than 5% of the number of nucleotides of the primer.

It is a preferred embodiment of this invention that the number of mismatches allowed for when virtually testing the amplification of unwanted products according to step 3 c) of the invention is less than seven.

It is especially preferred that the number of mismatches allowed for when virtually testing the amplification of unwanted products according to step 3 c) of the invention is less than five.

5

It is another preferred embodiment of this invention that the number of mismatches allowed for when virtually testing the amplification of unwanted products according to step 3 c) of the invention is less than three.

10

It is especially preferred in the scope of this invention that the number of mismatches allowed for when virtually testing the amplification of unwanted products according to step 3 c) of the invention is one.

15

It is also included in the scope of this invention to consider such primer molecules as being sufficiently similar to facilitate their binding to the template sequence, for which a template sequence can be found that differs in the number of nucleotides but is otherwise identical to the target sequence. When the alignment of the primer and the template sequence leads to a gap of up to 20% of the nucleotides of one sequence, preferably of the primer sequence, this shall still be considered to be sufficient for binding and hence potentially leading to the amplification of an unwanted product. Therefore these primers also need to be tested with the means of virtual PCR (for example with a program like e-PCR). Only if this test reveals the virtual amplification of an unwanted product caused by the combination of two primers, the according primer pairs are excluded from the set of selected pairs.

20

25

30

35

It is preferred that the number of nucleotides creating one gap, in one of the sequences, when aligning the primer molecule sequence with the template sequence, allowed

for when virtually testing the amplification of unwanted products according to step 3 c) of the invention is less than 20% of the number of nucleotides of the primer molecule.

5

It is also preferred that the number of nucleotides creating one gap, in one of the sequences, when aligning the primer molecule sequence with the template sequence, allowed for when virtually testing the amplification of unwanted products according to step 3 c) of the invention is less than 10% of the number of nucleotides of the primer molecule.

It is preferred that the number of nucleotides creating one gap, in one of the sequences, when aligning the primer molecule sequence with the template sequence, allowed for when virtually testing the amplification of unwanted products according to step 3 c) of the invention is less than 5% of the number of nucleotides of the primer molecule.

20

Both of these situations, mismatching due to an alternative nucleotide or no-matching due to a missing nucleotide, are meant to be covered in the expression describing those primer molecules that will eventually be selected : "said primer molecules are characterized in that every combination of two primer molecules, under conditions allowing for one or more base mismatches per primer, does not lead to the amplification of an unwanted product when virtually tested using the treated and the untreated sample nucleic acids as template".

25

30

It is also preferred that the primer molecules that exceed a pre-specified melting temperature when binding to the template have to be virtually tested for amplification of unwanted products using the treated and the untreated

35

ted sample nucleic acids as template according to step 3
c) of the method.

5 The basic problem of finding a primer specific enough to
give only one product on the little complex bisulfite
DNA, is finally solved by testing each potential primer
pair for hybridization across the whole bisulfite con-
verted human genome. This requires translating the whole
10 human genome sequence information virtually into its bi-
sulfite treated version before performing a similarity
search against the primer pairs, which is based on a
method like the so called e-PCR (Schuler G.D. (1997) Se-
quence Mapping by electronic PCR. Genome Research 7(5):
541-550). However, as the bisulfite conversion results in
15 two no longer complementary strands this virtual hybridi-
zation test needs to be done against both bisulfite con-
verted strands. In addition in most cases the template
DNA is contaminated with unconverted genomic DNA. To also
exclude unwanted amplification on the unconverted DNA as
20 template, the same hybridization test has to be performed
a third time using the whole human genome sequence as a
template.

25 Therefore it is a preferred embodiment of this invention
that the ability of said primer molecules to amplify an
unwanted product is tested by means such as electronic
PCR.

30 In the last step of the method said amplified target nu-
cleic acid gets detected by any means standard to one
skilled in the art.

In a preferred embodiment of this method the set of
primer molecules is comprised of at least two primer
35 molecules but not more than 64 primer molecules, given

the number is a multiple of 2; in other words, the set is comprised of 1-32 primer pairs.

5 In another preferred embodiment of this method the set of primer molecules is comprised of between 2 and 32 primer molecules, given the number is a multiple of 2; in other words the set is comprised of 1-16 primer pairs.

10 In a preferred embodiment of this method, said primer molecule comprises at least one nucleotide within the last three nucleotides from the 3' end of the molecule, wherein said nucleotide is complementary to a nucleotide of the target sequence that, as a result of the treatment performed in step 2) of the invention, changed its
15 hybridization behavior.

It is a preferred embodiment of this method, that said primer molecule comprises at least one nucleotide within the last three nucleotides from its 3' end that is complementary to a nucleotide of the target sequence that
20 was converted by the treatment performed in step 2 of the method to another base exhibiting an alternative base pairing behavior.

25 In an especially preferred embodiment said nucleotide is a cytosine prior to the treatment that converts unmethylated cytosines. In a preferred embodiment said treatment is bisulfite treatment. Said primer molecule comprises at least one nucleotide within the last three nucleotides
30 from the 3' end of the molecule, wherein said nucleotide is complementary to a cytosine, that was converted by bisulfite treatment to another base exhibiting the base pairing behavior of thymine.

35 This is to exclude binding of said primer molecules to the remaining untreated or un-sufficiently treated nu-

cleic acids, which might still serve as template nucleic acid in the PCR.

Furthermore it is a preferred embodiment of this invention that said primer molecules do not form loops or hairpins on their own or with each other.

In another preferred embodiment of the method said primer molecules do not form dimers with each other.

In the text the word 'hairpin' is taken to mean a secondary structure formed by a primer molecule when the 3' terminal region of said nucleic acid hybridizes to the 5' terminal region of said nucleic acid forming a double stranded stem structure and wherein only the central region of the primer is single stranded.

As described in the text the word 'loop' refers to a secondary structure formed by a primer molecule when two or more nucleotides of said molecule hybridize thereby forming a secondary structure comprising a double stranded structure one or more base pairs in length and further comprising a single stranded region between said double stranded region.

The binding of a primer molecules 3' end to any part of a second primer molecule in the set needs to be avoided. Otherwise the polymerase would extend the first primer using the second primer as template, which would lead to a new unwanted product, an extended primer, or rather a primer-hybrid, which would serve as the preferred template for the next round of the polymerase chain reaction and thereby prevent a sufficient amplification of the wanted product.

Therefore it is another preferred embodiment of this method that each of said primer molecules is characterized in that the last at least 5 bases at the 3' end of said primer molecule are not complementary to the sequence of any other primer molecule in the set.

It is also preferred that said primer molecules do not bind to nucleic acids which prior to treatment of step 2 contained a 5'-CG-3' site. This would lead to a binding of the primers to bisulfite treated nucleic acids, specifically depending on their cytosines methylation status. A CG corresponding primer would bind to the treated methylated version only, whereas a primer corresponding to TG would bind to the treated unmethylated version of these nucleic acids only. It is therefore preferred that said primer molecules do not contain nucleic acid sequences complementary or identical to nucleic acid sequences which prior to treatment of step 2 contained a 5'-CG-3' site.

In a preferred embodiment of this method said primer molecules are of a specified size range.

It is especially preferred that these primers are comprised of 16-50 nucleotides.

In a preferred embodiment of this method said primer molecules do not comprise sequences that are complementary to regions of the target nucleic acids that contained specified restriction enzyme recognition sites prior to the treatment that altered the unmethylated cytosines base pairing behavior. It is preferred that said primers are complementary to target sequences which prior to the treatment performed in step 2 of the invention did not contain specified restriction enzyme recognition sites.

By selecting for the right primer molecules also the amplificate's sequence is determined. That is why it has to be taken into account to only use those primer molecules that lead to amplification of nucleic acids containing a reasonable high number of CpG sites to be analyzed. Due to the treatment of step 2 of this invention these CpG sites, depending on the methylation status of the cytosine, are converted and will therefore either appear as CG dinucleotides or as TG dinucleotides in the amplificate.

It is preferred that said primer molecules amplify regions of nucleic acids that prior to bisulfite treatment comprise of more than eight 5'-CG-3' sites also referred to as CG dinucleotides.

It is also preferred that said primer molecules amplify regions of nucleic acids that prior to bisulfite treatment comprise of more than six 5'-CG-3' sites also referred to as CG dinucleotides.

It is also preferred that said primer molecules amplify regions of nucleic acids that prior to bisulfite treatment comprise of more than four 5'-CG-3' sites also referred to as CG dinucleotides and finally it is especially preferred that said primer molecules amplify regions of nucleic acids that prior to bisulfite treatment comprise of more than two 5'-CG-3' sites also referred to as CG dinucleotides.

Said primer molecules lead to amplificates within a specified size range.

It is a preferred embodiment of this sequence that said primer molecules lead to amplicates which are comprised of at least 50 bp but not more than 2000 bp.

- 5 Especially preferred are primer molecules that lead to amplicates which are comprised of at least 80 bp but not more than 1000 bp.

10 Furthermore a method is preferred wherein said primer molecules lead to amplicates of treated nucleic acids which prior to the treatment which altered the unmethylated cytosines base pairing behavior did not contain restriction enzyme recognition sites. Said primer molecules lead to amplicates that are amplified regions of the
15 treated nucleic acids which prior to the treatment performed in step 2) of the method did not contain specified restriction enzyme recognition sites.

20 A further subject of this invention is a method on how to produce said primer molecules. The main step of producing a primer molecule is determining its sequence. In the following the phrase "primer design" will be used instead of primer production, whenever it is referred to the step of determining said specific primer sequences. Designing
25 primer molecules is a process which as such is well known to scientists skilled in the art. The programs usually used for this purpose are such as PRIMER3 or OSP (Rozen S and Skaletsky H (2000) PRIMER3 on the WWW for general users and for biologist programmers. Methods Mol Biol 132: 365-386; Hillier L and Green P (1991) OSP: A computer
30 program for choosing PCR and DNA sequencing primers. PCR Methods and Applications 1: 124-128). Other primer design systems (like described in EP-A 1136932) are often based on those commonly known programs.

An embodiment of this invention takes advantage of using a program like PRIMER3 first, to then add a number of steps that finally result in an advanced method of designing primers that are specifically useful for amplifying sequences of low complexity.

In the first step of this method for designing specific primer molecules for nucleic acids of low complexity, primer pairs that amplify single products are selected by applying standard tools of primer design known in the art, like for example the program PRIMER3 (Rozen, S and Skaletsky, H (2000) Methods Mol Biol 132: 365-386).

In the second step of the method said primer pairs are tested whether or not one of its primer molecules when hybridizing to any other primer molecule in the set exceeds a specified threshold melting temperature T_M . If this is the case the primer pair that comprises of said primer is excluded from the set of potentially combined pairs.

In the third step of the method the number of previously selected primer pairs, is reduced to a smaller number by implementing as new criteria a measure for the primer sequence's complexity. Primer pairs that consist of a primer molecule which does not meet said criteria are excluded.

The basic problem of finding a primer specific enough to give only one product on the little complex bisulfite DNA, is finally solved by testing each potential primer pair for hybridization across the whole bisulfite converted human genome. This requires translating the whole human genome sequence information virtually (as in "in silico") into its treated, for example bisulfite treated, version before performing a similarity search against the

primer pairs, which is based on a method like the so called e-PCR (Schuler G.D. (1997) Sequence Mapping by electronic PCR. Genome Research 7(5): 541-550). However, as the bisulfite conversion results in two different versions of the double helix whose sense and anti-sense strands are no longer mutually complementary, this in silico amplification needs to be performed on both bisulfite converted versions of the genome. In addition in most cases the template DNA is contaminated with unconverted genomic DNA. It cannot be excluded that single cytosines or longer runs of DNA remain unconverted or are only converted incompletely by the bisulfite treatment. To also exclude unwanted amplification of the unconverted DNA as template, the same hybridization test has to be performed a third time using the whole human genome sequence as a template.

As this is quite some effort and requires time (CPU time) this is the fourth and last step of this design method, that is absolved prior to the final testing in a "wet", lab based, experiment.

In addition to improve the specificity of said primer molecules the stringency of the selection criteria is increased: Some standard primer design tools disqualify a primer if in the template sequence, a second identical sequence, besides the target sequence, can be found. That way mispriming at rather stringent hybridization conditions is avoided. This mispriming would not necessarily lead to an additional unwanted product, but would lead to the dilution of the primer molecules available for amplification. This selection has been performed in step one already (for example by PRIMER3). However, due to the higher probability of a bisulfite primer molecule to mismatch with non-identical bisulfite treated DNA, there is still a chance for said primer molecules to misprime even

when up to 20% of the nucleotides of the primer sequence differ. Therefore it is claimed in this invention to only use primer molecules for which not even a weak sequence homology can be found. However, this would exclude primer molecules unnecessarily. Therefore they are only excluded if two primer molecules match to the template and amplify an unwanted product. This test is performed by means as, for example, the Electronic PCR. Electronic PCR (e-PCR) is an in silico virtual PCR carried out in order to assess the suitability of primers prior to in vitro PCR.

In the fourth step of the method on how to design these primers it is therefore tested whether there are any regions of the template nucleic acid, said template being comprised of the sense and the anti-sense strand of the treated and the untreated nucleic acids, that are identical in sequence with the primer molecule to more than 80% and if those primer molecules are able to amplify an unwanted product. If this is the case, the primer pair comprising said primer molecule is excluded from the selection.

The template nucleic acid is comprised of the treated template nucleic acid and the untreated template nucleic acid. The treated nucleic acid in itself is comprised of a two strands which after treatment are not complementary to each other anymore. This virtual testing for example can be performed as described by Gregory Schuler in his article (cited above) about sequence mapping by "Electronic PCR". The primer pairs remaining can be used to specifically amplify regions of nucleic acids of low complexity, which is the aim of this invention. Hence step 4 of the design method is the virtual testing of each possible primer pair combination, under pre-specified conditions at a stringency allowing for one or more base pair mismatches, as to whether no unwanted nucleic acids are

amplified. Said virtual testing is carried out upon both untreated and treated nucleic acids. The wording "possible combinations" refers to all combinations that are possible within a set of primer pairs to be used in one amplification reaction vessel.

In a preferred embodiment an additional step is added following the virtual testing, which is testing in a lab based single PCR assay all those pairs that remained, whether the desired amplificate can be obtained or not. If that is the case, the chosen pairs can be used to specifically amplify those regions of nucleic acids of low complexity according to the method as described before.

In a specially preferred embodiment the first step of the design method is characterized as selecting a pool of possible primer pairs per amplificate by means of a standard PCR primer design program using said nucleic acids as template that have been masked for repeats and SNPs considering the following factors:
length of amplificate, length of primer, melting temperature of the primer molecule, dimer formation parameters, loop formation parameters, exclusion of unidentified or ambiguous nucleotides in the primer sequence, exclusion of restriction enzyme recognition sites.

In a preferred embodiment of this invention this measure of complexity is a measure of linguistic complexity as defined by Bolshoy et al. (see above). Those primer pairs are excluded from the previously selected ones, which comprise of one primer that doesn't reach a set level of this linguistic complexity.

In another preferred embodiment of this invention this measure of complexity is a measure of Shannon entropy (as described before).

In an especially preferred embodiment of this design method, prior to performing step d) the additional step of excluding primer pairs from the remaining primer pairs which consist of a primer molecule that comprises of at least one CpG site, is carried out.

In an especially preferred embodiment of this method according to the design of said primers, prior to performing step d) the additional step of excluding primer pairs from the remaining pairs when one of its primer molecules does not contain at least one nucleotide within the last three nucleotides from the 3' end of the molecule wherein said nucleotide is complementary to a nucleotide of the target sequence that was converted to a different nucleotide by bisulfite treatment, is carried out.

In an especially preferred embodiment of this method according to the production of said primers, prior to performing step d) the additional step, of excluding primer pairs from the remaining primer pairs which amplify a nucleic acid that did not prior to treatment with bisulfite contain a minimum of two CpG sites, is carried out.

In an especially preferred embodiment of this method according to the production of said primers, prior to performing step d) the additional step of excluding primer pairs from the remaining primer pairs when one of its primer molecules contains more than 5 bases at its 3' end that are complementary to any other primer molecules sequence in the set, is carried out.

In an especially preferred embodiment of this method according to the production of said primers, prior to performing step d) the additional step of excluding from the remaining primer pairs those pairs, which comprise of one

primer molecule that in combination with another primer molecule in the set amplifies an unwanted product, when virtually testing according to step 3 c) of the amplification method under conditions allowing for a number of mismatching nucleotides of 20% of the number of nucleotides of the primer molecule, is carried out.

In an especially preferred embodiment of this method according to the production of said primers, prior to performing step d) the additional step of excluding from the remaining primer pairs those pairs, which comprise of one primer molecule that in combination with another primer molecule in the set amplifies an unwanted product, when virtually testing according to step 3 c) of the amplification method under conditions allowing for a number of nucleotides creating one gap, when aligning the primer molecule sequence with the template sequence, of up to 20% of the number of nucleotides of the primer molecule, is carried out.

In an especially preferred embodiment of this method according to the production of said primers, prior to performing step d) the additional step of excluding from the remaining primer pairs those pairs, which comprise of one primer molecule that in combination with another primer molecule in the set amplifies an unwanted product, when virtually testing according to step 3 c) of the amplification method under conditions allowing for four or less mismatching base pairs, is carried out.

In an especially preferred embodiment of this method according to the production of said primers, prior to performing step d) the additional step of excluding from the remaining primer pairs those pairs, which comprise of one primer molecule that in combination with another primer molecule in the set amplifies an unwanted product, when

virtually testing according to step 3 c) of the amplification method under conditions allowing for two or less mismatching base pairs, is carried out.

5 The following example is intended to illustrate the invention :

Example

10 Here we present experimental data that shows that multiplex PCRs designed with a tool according to this invention are more successful compared to multiplex PCRs not designed in this manner.

15 It is the aim of the experiment to amplify 40 different nucleic acids. The genomic regions of interest are given in the sequence protocol (SEQ ID 41-80). These genomic sequences were translated into their bisulfite converted versions and served as templates for amplification of
20 specific regions with the primer sequences described as follows.

Primer molecule pairs used for single PCRs were originally designed with the use of the standard primer design
25 program PRIMER3 (as mentioned in the description). The criteria used in that step will not be discussed in detail. This selection however provides several possible primer pairs per amplificate. Following the present invention these primer pairs were selected further, according to the following criteria:
30

- The restriction enzyme recognition site to be excluded from the genomic nucleic acid (which subsequent to bisulfite conversion becomes the template
35 for the PCR amplification step) is : GTTTAAAC.

- The minimum length of the primer molecule is 18 nucleotides. The maximum length is 27 nucleotides. Ideally the primer consists of 22 nucleotides.
- 5 • The minimum required measure of linguistic complexity is 0.2.
- The minimum melting temperature of a primer molecule is 54°C and the maximum melting temperature is 57°C.
- 10 The ideal melting temperature however is 55°C.
- The minimum length of an amplificate is 100 bp and the maximum length is 500 bp.
- 15 • The minimum number of CpG sites, that were present in the region of the nucleic acid, prior to bisulfite treatment, that was amplified is 4.
- The number of mismatch bases allowed for when virtually testing the primer pairs according to the invention for amplification of an unwanted product with the help of e-PCR (Electronic PCR) is 2.
- 20

The use of this invention, that is the use of either the design method, being the subject of the invention, and/or performing the steps of said method as described above (assuming a set size of 1) leads to the selection of the following 40 optimized primer molecule pairs:

TABLE 1:

primer sequence	amplificate identifier	SEQ ID	number indicating primer direction	starting position of primer in the bisulfite converted sequence of the ROI
AATCCTCCAAATTCTAAAAACA	2025	81	0	1816
AGGAAAGGGAGTGAGAAAAT	2025	82	1	2138

primer sequence	amplificate identifier	SEQ ID	number indicating primer direction	starting position of primer in the bisul- fite converted se- quence of the ROI
GGATAGGAGTTGGGATTAAGAT	2044	83	0	2070
AAATCTTTTTCAACACCAAAT	2044	84	1	2483
AACCCTTTCTTCAAATTACAAA	2045	85	0	1340
TGATTGGGTTTTAGGGAAATA	2045	86	1	1687
TTGAAAATAAGAAAGGTTGAGG	2106	87	0	1481
CTTCTACCCCAAATCCCTA	2106	88	1	1764
TGTTTGGGATTGGGTAGG	2166	89	0	2226
CATAACCTTTACCTATCTCCTCA	2166	90	1	2437
TTTTAGATTGAGGTTTTAGGGT	2188	91	0	101
ATCCATTCTACCTCCTTTTTCT	2188	92	1	598
GGAGGGGAGAGGGTTATG	2191	93	0	133
TACTATACACCCCCAAAACAA	2191	94	1	506
TTTTGGGAATGGGTTGTAT	2194	95	0	1628
CTACCCTTAACCTCCATCCTA	2194	96	1	1996
TTGTTGGGAGTTTTTAAGTTTT	2212	97	0	1711
CAAATTCTCCTTCCAAATAAAT	2212	98	1	2063
GTAATTTGAAGAAAGTTGAGGG	2267	99	0	1709
CCAACAATAAACAACCTCT	2267	100	1	2004
GGAGTTGTATTGTTGGGAGA	2317	101	0	1110
TAAAACCCAATTTTCACTAA	2317	102	1	1388
TTTGTATTAGGTTGGAAGTGGT	2383	103	0	1
CCCAAATAAATCAACAACAACA	2383	104	1	285
GATTTTTGGAGAGGAAGTTAAG	2387	105	0	789
AAAATAAAAACCAAACCCATA	2387	106	1	1169
TGGGGTTAGTTTAGGATAGG	2391	107	0	1353
CTTAAAAACACTAAACTTCTCAAA	2391	108	1	1750
TTTTTGATTGGGGTAGGTTT	2395	109	0	547
CCCAACTATCTCTCTCCTCTATAA	2395	110	1	1094
ATTAGAAGTGAAAGTAATGGAATTT	2401	111	0	381
TCAATTTCCAAAAACCAAC	2401	112	1	795

primer sequence	amplificate identifier	SEQ ID	number indicating primer direction	starting position of primer in the bisulfite converted sequence of the ROI
GGGATGGGTATTAGTTGTAAA	2453	113	0	1867
CCTTCACACAAAACACTACAAAA	2453	114	1	2139
TAATTGAAGGGGTTAATAGTGG	2484	115	0	1861
AAAACCAAAACCAAAACTAAAA	2484	116	1	2252
AGTGGATTTGGAGTTTAGATGT	2512	117	0	1016
AACAAAATAAAAACCTTCTCCCA	2512	118	1	1446
TAGGGGAAAAGTTAGAGTTGAG	2741	119	0	1413
CCCATTAACCCACAAAAA	2741	120	1	1888
ATTTTAGTTTGTGAAATGGGAT	2745	121	0	1685
TCTTAACCAATAACCCCTCAC	2745	122	1	2097
GTGGGTTTTGGGTAGTTATAGA	2746	123	0	1679
TAACCTCCTCTCCTTACCAA	2746	124	1	2163
TAGGATGGGGAGAGTAATGTTT	2747	125	0	972
ACAACTTATCCAACCTCCATTC	2747	126	1	1448
TCCCACAAAACCTAAACAATTA	2749	127	0	1370
AGGTTTTAGATGAAGGGGTTT	2749	128	1	1789
TTTGGAGGGTTTAGTAGAAGTTA	2751	129	0	88
CCCAATAATCACAAAATAAACA	2751	130	1	567
ATACAACCTCAAATCCTATCCA	2752	131	0	228
AGGGAGAAGGAAGTTATTTGTT	2752	132	1	712
GGAAGATGAGGAAGTTGATTAG	2755	133	0	1000
CCTACAACCCTATCCTCTAAAA	2755	134	1	1371
TTAGTAGGGGTGTGAGTGTTTT	2831	135	0	1313
CAAACAAAACCTTCTATCTCAACC	2831	136	1	1499
TTATAGGGTTGAGTTTGGGAT	2850	137	0	2100
TAAACAAACAACAAATCTTCCA	2850	138	1	2400
TGAAAATGAAGGTATGGAGTTT	2852	139	0	1262
TTAAAACCATATAATCCCTCCA	2852	140	1	1583
TATGTTTGGTTTTGTTTGGAGA	2859	141	0	1093
AACCCCATCACTTTTATTTCTT	2859	142	1	1491

primer sequence	amplificate identifier	SEQ ID	number indicating primer direction	starting position of primer in the bisulfite converted sequence of the ROI
GGGTGTAGAAGTGTTTAGGTTT	2861	143	0	2385
TTTCTCCCCTTACAACAATAAC	2861	144	1	2732
TCCCCTTCCAACATATCTCTC	2864	145	0	884
TGAGAGTGTTTTAGGGAAGTTT	2864	146	1	1175
AAAACCAAAACATAAACCAAAA	2867	147	0	1312
GATTAGGAGGGTTTGTTGAGAT	2867	148	1	1701
AATGGTTGATGATTTTGGTTT	2961	149	0	2039
ACTCTCTTCCCTATACCCCTAA	2961	150	1	2311
AGTTAGAAGAGGAGTTAGGATGG	3511	151	0	1340
TAATTTTCCAATACCCATTTTC	3511	152	1	1711
TGTTAGTAGAGTTTTAGGGAGGTT	3532	153	0	1135
ACACTACCTATCCTTACCCAC	3532	154	1	1592
TTTTTGTTTTTATGGGGTGTAT	3534	155	0	1909
TTAAATATCCCTTCCTTAACCA	3534	156	1	2385
TGGGTAGTATTTTTGTTGGTTT	3538	157	0	956
CCTAAAACTCTCTCATCCTCA	3538	158	1	1414
AGTGGTTTAGGAGTATTTGGTTA	3540	159	0	659
AACTCCCTCCATCTACAATATC	3540	160	1	1064

These primer pairs lead to the amplification of specific regions (amplificates Seq IDs 1- 40) of the bisulfite converted sequences of the genomic ROIs (Seq IDs 41- 80) of interest. The ROIs can be identified by the four digit number that specifies the ROI and the corresponding amplificate - as indicated in the following table.

TABLE 2:

SEQ ID	Class	Identifier	Kind of DNA	SEQ ID	Class	Identifier	Kind of DNA
1	amplificate	2025	bisulfite sequence	41	ROI	2025	genomic sequence

SEQ ID	Class	Identifier	Kind of DNA	SEQ ID	Class	Identifier	Kind of DNA
2	amplificate	2044	bisulfite se- quence	42	ROI	2044	genomic se- quence
3	amplificate	2045	bisulfite se- quence	43	ROI	2045	genomic se- quence
4	amplificate	2106	bisulfite se- quence	44	ROI	2106	genomic se- quence
5	amplificate	2166	bisulfite se- quence	45	ROI	2166	genomic se- quence
6	amplificate	2188	bisulfite se- quence	46	ROI	2188	genomic se- quence
7	amplificate	2191	bisulfite se- quence	47	ROI	2191	genomic se- quence
8	amplificate	2194	bisulfite se- quence	48	ROI	2194	genomic se- quence
9	amplificate	2212	bisulfite se- quence	49	ROI	2212	genomic se- quence
10	amplificate	2267	bisulfite se- quence	50	ROI	2267	genomic se- quence
11	amplificate	2317	bisulfite se- quence	51	ROI	2317	genomic se- quence
12	amplificate	2383	bisulfite se- quence	52	ROI	2383	genomic se- quence
13	amplificate	2387	bisulfite se- quence	53	ROI	2387	genomic se- quence
14	amplificate	2391	bisulfite se- quence	54	ROI	2391	genomic se- quence
15	amplificate	2395	bisulfite se- quence	55	ROI	2395	genomic se- quence
16	amplificate	2401	bisulfite se- quence	56	ROI	2401	genomic se- quence
17	amplificate	2453	bisulfite se- quence	57	ROI	2453	genomic se- quence

SEQ ID	Class	Identifier	Kind of DNA	SEQ ID	Class	Identifier	Kind of DNA
18	amplificate	2484	bisulfite se- quence	58	ROI	2484	genomic se- quence
19	amplificate	2512	bisulfite se- quence	59	ROI	2512	genomic se- quence
20	amplificate	2741	bisulfite se- quence	60	ROI	2741	genomic se- quence
21	amplificate	2745	bisulfite se- quence	61	ROI	2745	genomic se- quence
22	amplificate	2746	bisulfite se- quence	62	ROI	2746	genomic se- quence
23	amplificate	2747	bisulfite se- quence	63	ROI	2747	genomic se- quence
24	amplificate	2749	bisulfite se- quence	64	ROI	2749	genomic se- quence
25	amplificate	2751	bisulfite se- quence	65	ROI	2751	genomic se- quence
26	amplificate	2752	bisulfite se- quence	66	ROI	2752	genomic se- quence
27	amplificate	2755	bisulfite se- quence	67	ROI	2755	genomic se- quence
28	amplificate	2831	bisulfite se- quence	68	ROI	2831	genomic se- quence
29	amplificate	2850	bisulfite se- quence	69	ROI	2850	genomic se- quence
30	amplificate	2852	bisulfite se- quence	70	ROI	2852	genomic se- quence
31	amplificate	2859	bisulfite se- quence	71	ROI	2859	genomic se- quence
32	amplificate	2861	bisulfite se- quence	72	ROI	2861	genomic se- quence
33	amplificate	2864	bisulfite se- quence	73	ROI	2864	genomic se- quence

SEQ ID	Class	Identifier	Kind of DNA	SEQ ID	Class	Identifier	Kind of DNA
34	amplificate	2867	bisulfite se- quence	74	ROI	2867	genomic se- quence
35	amplificate	2961	bisulfite se- quence	75	ROI	2961	genomic se- quence
36	amplificate	3511	bisulfite se- quence	76	ROI	3511	genomic se- quence
37	amplificate	3532	bisulfite se- quence	77	ROI	3532	genomic se- quence
38	amplificate	3534	bisulfite se- quence	78	ROI	3534	genomic se- quence
39	amplificate	3538	bisulfite se- quence	79	ROI	3538	genomic se- quence
40	amplificate	3540	bisulfite se- quence	80	ROI	3540	genomic se- quence

The second task in this example is to select from these
 40 primer pairs those pairs which can be combined in five
 multiplex PCRs to amplify eight targets simultaneously.

The following steps, as disclosed in the invention, are
 performed for selection of those subsets:

- The melting temperature of any combination of two of those primer molecules hybridizing to each other taking part in one multiplex experiment must be below 20°C.
- The last seven nucleotides from the 3' end of every primer molecule in a subset is used to check if those are complementary and/or binding to any other primer molecules' sequence used in the set.

- The number of mismatch bases allowed for when virtually testing the primer pairs for amplification of an unwanted product is 2. For this step every possible combination of 16 primer molecules in one subset is checked for its ability to amplify an unwanted product. This is done by means of e-PCR (electronic PCR).

10 Having performed all these steps results in the selection of three different optimized sets of primer molecule pairs that can be used in multiplex PCRs. These sets are in the following described as a set of numbers. Each number refers to a specific amplificate and therefore also
15 to a single primer pair (out of the list given above) which proved to be able to specifically amplify said nucleic acid in a single PCR experiment.

TABLE 3:

optimized set 1								
8plex1	2194	2191	2391	2025	2961	3540	2861	2188
8plex2	2484	2106	2401	2850	3532	2044	2512	2852
8plex3	2453	2741	2867	2755	2267	2387	2864	2317
8plex4	2859	2383	2752	2747	2751	3511	2212	2746
8plex5	3534	2395	2745	3538	2749	2166	2831	2045
optimized set 2								
8plex1	2166	2212	3511	2383	2745	2859	3534	2861
8plex2	2749	2191	2751	2395	2961	2512	2831	3538
8plex3	2850	2025	2188	2317	2391	2852	3540	2194
8plex4	2106	2387	2867	2864	2401	2747	2746	2453
8plex5	2044	2484	2267	2755	2752	2741	2045	3532
optimized set 3								
8plex1	2194	2391	2191	2749	2745	3538	2861	2961
8plex2	2166	2188	2859	2212	2864	2746	2383	2752
8plex3	2484	2401	2850	2852	2512	2755	2106	2044
8plex4	2867	2453	3532	2025	2741	2267	2317	2387
8plex5	3511	3534	2751	2747	2395	3540	2831	2045

Without the use of said invention, the selection would have been performed randomly and tested for successful application later. Three randomly chosen subsets are shown here.

TABLE 4:

random set 1								
8plex1	2191	2194	2267	2741	3534	3511	2749	2747
8plex2	2391	2484	2867	2852	2453	2512	2025	3538
8plex3	2746	2212	2755	2045	2044	2188	2961	2864
8plex4	2831	2383	3540	2859	2861	2395	2401	2317
8plex5	2106	2751	2387	2745	2752	3532	2850	2166
random set 2								
8plex1	2045	2106	2212	2745	2044	2749	2752	2391
8plex2	2025	2831	2401	3540	2395	2484	2453	2961
8plex3	2194	2859	2746	2512	2267	2864	2861	2751
8plex4	2383	2166	2747	2387	3532	2741	2867	2852
8plex5	3534	2755	2850	2317	2191	3538	3511	2188
random set 3								
8plex1	2484	2850	2741	2747	2755	2745	2025	2746
8plex2	2383	3534	2861	2751	2749	2391	2188	2191
8plex3	2194	3538	2512	2961	2864	2867	2831	3532
8plex4	3511	2045	2387	2212	2166	2267	3540	2401
8plex5	2395	2317	2859	2453	2852	2106	2752	2044

The sequences of all of those amplificates and the according primers are given in the sequence protocol (primers SEQ IDs 81-160; amplificates SEQ IDs 1-40). SEQ IDs refer to the internal numbers used in these tables as is shown in TABLES 1 and 2.

To show if the use of the design method described herein was superior to the common method of selecting primers for simultaneous amplification randomly said multiplex PCRs were performed. This example hereby demonstrates the advantage of the method which is subject of the invention:

A total of 40 amplicates (with lengths ranging from 187 - 499 bp) were partitioned into five 8-plex PCRs using either of two strategies.

5 First: the grouping was based on the invention using said "optimised sets" ("designed group").

Second: the grouping was done without using the selection criteria established by this invention using the "random
10 sets" ("control group").

Whether such grouping can improve the success rate of mPCRs was subsequently tested experimentally by comparing the number of true and false positives and false nega-
15 tives for each of the two classes.

Each of the five mPCRs (multiplex PCRs) contained 8 primer pairs specific for 8 amplicates with one primer of each pair being labeled with a Cy-5 fluorescent tag.
20 Only fragments that performed successfully in sPCR (singleplex PCR) using bisulfite-modified human DNA from whole blood were included in this study. Isomolar primer concentrations were used in a 20µl PCR reaction volume and cycling was done for 42 cycles using a 96-well micro-
25 titer plate thermocycler.

Group assignments for the "optimized" and "random" groups were done in triplicate and all mPCRs were run at the same time such as to minimize experimental variation in
30 PCR performance.

A mixture of the amplicates that were expected to be generated in a specific mPCR reaction but were generated in eight corresponding sPCR reactions was called sPCR-
35 pool. Electrophoresis of sPCR-pool amplicates and mPCR amplicates was done simultaneously using the ALFexpress

system (Amersham Pharmacia). In order to obtain the best comparability for mPCRs with their respective sPCR standard, these products were electrophoresed next to each other on the gels.

5

Figures 1 and 2 show examples of these results as electropherograms, given as ALFexpress output files.

10

Success or failure scoring for each mPCR was based on assessing the number of generated or absent fragments compared to their respective pool of sPCR fragments. Only fragments with peak areas equal or larger than 8% of the largest peak within one electropherogram were included into the analysis.

15

Figure 1 illustrates a result of an 8-plex PCR based on a primer combination from the "optimized set". The top graph in the figure shows peaks of size standards only. The second graph in the figure shows the electrophoresed mixture of the products from 8 singleplex PCRs. The third graph shows the products resulting from a multiplex PCR employing one of the optimized sets of primer combinations. By comparing these graphs it becomes visible that, in this specific example, there is only one false negative (FN) and three false positives (FP), whereas there are eight true positives (TP).

25

Figure 2, however, illustrates a result of an 8-plex PCR based on a primer combination from the "control set". The top graph in the figure shows peaks of size standards only. The second graph in the figure shows the electrophoresed mixture of the products from 8 singleplex PCRs. The third graph shows the products resulting from a multiplex PCR employing one of the randomly chosen sets, as is the state of the art. This graph clearly shows that, there are eight false negative and six false positive

30

35

peaks, whereas there is only one true positive. Hence, for this specific example we have demonstrated the superiority of the design method.

5 A more comprehensive view on the results is given in **Figures 3 and 4.**

By applying the Wilcoxon rank sum test for the determination of false positives or false negatives as follows, it
10 becomes evident that the optimized set resulted in a more reliable amplification experiment:

data: False negatives (FN)
p-value = 0.02602 rejection of null hypothesis
15 null hypothesis (H0): true if median of designed set equal or greater than of control set alternative hypothesis (H1): true if median of designed set less than of control set

20 data: False positives (FP)
p-value = 0.06711 rejection of null hypothesis
null hypothesis (H0): true if median of designed set equal or less than of control set
alternative hypothesis (H1): true if median of designed
25 set greater than of control set

data: True positives (TP)
p-value = 0.02146 rejection null hypothesis
null hypothesis (H0): true if median of designed set
30 equal or less than of control set
alternative hypothesis (H1): true if median of designed set greater than of control set

Figure 3 illustrates a summary of several such comparisons (as described in detail above). Six diagrams are
35 shown, that illustrate the numbers of false positives

(FP), false negatives (FN) and true positives (TP) for a number of 18 experiments. In the top row of figure 3 the results for experiments that employed the design method are shown whereas in the lower row results from experiments are shown, that did use the conventional method of random selection.

At the x-axis the occurrence of an event (like a false positive) per 8plex is given whereas the values of the y-axis indicate the frequency of an event like this occurring within the number of experiments performed.

For example, in the diagram title FN, a y-value of 0 indicates that the event did not occur in a single experiment, a y-value of four indicates that the according number of occurrences given as the x-value was found in four experiments (out of the 18 experiments considered for these analyses). The x-value indicates what kind of occurrence is counted; a x-value of three in this diagram indicates the occurrence of three false negatives. A data point with an x-value of 0 and an y-value of 9 means, that in the set of mPCR results considered, nine experiments showed 0 false negatives.

Figure 4 gives all of the data from the 18 multiplex PCR experiments of this example in one table. The letter A, heading the four columns presented on the left side, is indicating the results from multiplex PCRs of the designed group using the five optimized sets of primer pairs that have been designed and selected according to the invention. The letter C is indicating the results from multiplex PCRs of the control group using the five randomized sets of primer pairs.

The first column lists the identifying numbers of the experiments, the second column gives the numbers of true

positives (TP) within this experiment, the third column gives the numbers of false positives (FP) and the last column gives the numbers of false negatives (FN).

5 The average false negative rate (\emptyset FN) of the optimized group is significantly lower than in the control group. Complementary the average true positive rate (\emptyset TP) is significantly higher. The average false positive rates (\emptyset FP) of the two sets do not differ from each other sig-
10 nificantly.

This is due to the high deviation of false positives observed between individual ALFexpress analysis runs. Those 36 sets of amplicates have been analyzed on two separate gel runs. These runs were not designed to simply duplicate the results, but could be used to analyze whether
15 the average TP, FP and FN rates are similar, independent of the run, and the sets chosen. Only three of those sets have been duplicated, as indicated by the letters a and b for sets 11, 21 and 23. It turned out that the rate of
20 true positives as well as the rate of false negatives averaged over 18 sets per run were highly reproducible, 6.83 versus 7.33 and 1.44 versus 1.39 respectively. However, the rate of false positives was determined as 4.11
25 in the first run and 7.61 in the second run.

Taken together, it could be concluded that the overall success rate of amplifying 40 fragments within 5 groups of 8plex PCRs was significantly increased when the primer
30 grouping was based on the method being subject of this invention compared to an arbitrary primer grouping. The improved success rate of only 11% failures versus 24% in the random control group clearly becomes relevant when much larger numbers of mPCRs have to be established as is
35 the case in a high throughput laboratory.